In the United States Patent and Trademark Office

Applicants: Steven Curtis Zicker *et al.*	Examiner: Z. Vakili
Serial No.: 09/978,127	Art Unit: 1614
Filing Date: October 16, 2001	Confirmation No.: 3786
For: **COMPOSITION AND METHOD**	Attorney Docket No.: 6493-02-HL

DECLARATION UNDER 37 C.F.R. § 1.131 OF DR. STEVEN C. ZICKER

1. I am a co-inventor of pending Claims 39 and 44-47 of the above-captioned case.

2. I am aware that art has been cited against the claims by the Examiner in a final rejection mailed December 4, 2007.

3. I have been told that the earliest effective date of Harper publication WO 00/44375 is January 31, 2000. I also understand the earliest effective date of Hamilton patent US 6,335,361 is November 3, 1999, based on priority of Provisional Application No. 60/163,352 filed that date.

4. As demonstrated in the attachments, the invention that is the subject matter of Claims 39 and 44-47 was made on a date earlier than November 3, 1999. First, I demonstrate that a dog food containing four antioxidants (Vitamin E, Vitamin C, alpha-lipoic acid, and L-carnitine) was conceived and formulated before November 3, 1999. Then I show that the idea to use the 4-antioxidant composition in the claimed method was also made earlier than that date.

Composition with four antioxidants

5. The three pages attached as Exhibit A, B, and C are production batch sheets, showing production of a dog food containing Vitamin E, Vitamin C (ascorbic acid), L-carnitine, and alpha lipoic acid.

6. The Exhibits give the recipe for Formula No. 166668 described under Formula Description as "NMSENIOR TEST + CARNIT/TAU." Exhibit A is a "Make Sheet for Grain Mix," Exhibit B is a "Make Sheet for Grain Premix," and Exhibit C is a "Make Sheet for Topical." They indicate that I requested the formulations to be produced in the Hills Experimental Food Laboratory (EFL).

7. The Make Sheet for Grain Mix (Exhibit A) relates that the composition makes up 94.65% of the finished product. The composition of the Make Sheet for Grain Premix (Exhibit B) makes up 2.36% by weight of the final product. The composition on the Make Sheet for Topical (Exhibit C) makes up 5.35% by weight of the finished product. The numbers for the premix are a component of the grain mix which totals 94.65%. Therefore the grain mix is grain (92.29%) grain premix (2.36%) and topical (5.35%) which totals 100%.

8. The date redacted following "printed by Steven Zicker" at the upper left of the Exhibits is earlier than November 3, 1999. The Exhibits demonstrate that the formulas were conceived on or before the redacted date. The date is further corroborated by the initials LH of the Plant Manager, shown in redacted form at the top right of Exhibit B. The redacted date next to the Plant Manager's initials is also earlier than November 3, 1999.

9. The Exhibits also contain redacted dates next to initials at the lower right. The initials belong to the technicians who made the product in the EFL and to the Plant Manager LH who also signed off.

10. I am confident of the dates on the attached Exhibits. At the time of the invention, experimental formulas were computed in Agridata, a commercially-available software program which was in use at Hill's in 1999 and earlier. Experimental formulas were first stored in my own personal folder. If the formula was to be made into a physical product, the following steps were taken:

(1) Save the formula as a production formula in Agridata.

(2) Export the formula to the Test Information Management System (TIMS) proprietary software for assignment of a formula number.

(3) Once the formula number was assigned, a batch sheet could be produced for the EFL to make the food.

(4) The batch sheet would be checked by the Plant Manager and scheduled for production.

(5) The product was produced in the EFL, bagged and samples saved for analysis.

(6) Analytical information was reported and downloaded into the TIMS with the associated formula number and date of production of the food.

The Exhibits show a Formula number of 16668 was assigned by the Agridata System. The attached Make Sheets for the product were checked by the Plant Manager, as indicated by his initials LH in the upper right-hand corner of the Grain Premix Make Sheet. The "print date" redacted next to my name in the upper left hand corner of the Exhibits is a reliable indication that the compositions were conceived on or before that date.

11. The Make Sheet for Grain Premix (Exhibit B) indicates the percent by weight of the diet of the four antioxidants: Vitamin E, Vitamin C, carnitine, and alpha lipoic acid. The ingredient listing indicates that the diet contains 0.241% of Vitamin E (50% by weight), 0.029% of Vitamin C (ascorbic acid), 0.25% by weight of 10% L-carnitine, and 0.014% by weight of alpha lipoic acid. These percentages by weights translate to the following ppm values, taking into account the percent active of the respective ingredient.

component	calc'd from recipe	measured in finished product
Vitamin E	1205 ppm	935 ppm
Vitamin C	290 ppm	210 ppm
carnitine	257 ppm	266 ppm

alpha lipoic acid	140 ppm	136 ppm

The middle column is the amount calculated from the make sheets. The right hand column gives the results of the analytical measurement made on the actual batch 16668. These values are on an as is basis prior to correction for water content of the food. In addition, processing losses may occur during the extrusion process so that final analyzed values will be different than computer projection from the formulation alone. The variations of the measured from the calculated values in the table are completely normal and are expected.

12. "Carnitine" appearing in the Make Sheets and in the table above is L-carnitine. We only use L-carnitine in animal formulations. D-carnitine is toxic and our specification sheets are for L-carnitine only. Any other reference to carnitine in our databases or analyses means we are talking about L-carnitine.

13. In addition to the above levels of antioxidants, the finished product based on the three Make Sheets is a complete diet for an adult dog. The nutrients as projected by the computer program are sufficient to support the claims for a maintenance food for dogs above 1 year of age as per AAFCO (Association of American Feed Control Officials) recommendations.

14. I conclude that, based on the three Make Sheets attached as Exhibit A, B, and C, an embodiment of a pet food falling within the scope of Claims 39 and 44-47 was made on a date earlier than the November 3, 1999 earliest effective date of the art cited against our claims.

Method of use of the composition

15. At the time the composition was made, we intended to use it in the claimed method of inhibiting the loss of learning ability or increasing the learning ability of an aged companion pet. We had thus fully conceived the invention of the

claims, and all that remained was for our contract lab to carry out the testing at our instruction.

16. We carried out the animal experiments at the Lovelace Respiratory Research Institute in New Mexico (LRRI). Appendix D is a document we submitted to LRRI for approval of the project according to USDA regulations. Those regulations require that all facilities doing animal testing have an Institutional Animal Care and Use Committee (IACUC) which reviews the projects for conformance to safety, health and other concerns.

17. The date of Appendix D is earlier than November 3, 1999. This is reflected in several places in the document. On the first page in the box marked "For Office Use Only," the date received and the approval date as well as the proposal number are redacted. The date received and approval date are earlier than November 3, 1999. The proposal number is redacted because it contains information about the fiscal year in which the proposal was made. The revision date of the Form on the bottom of pages 1-4 is redacted. The revision date is earlier than November 3, 1999. The last page of Appendix D contains a signature by Dr. David G. Burt of the LRRI. The redacted date next to his signature is earlier than November 3, 1999. Also on the last page, the review date and its corrections next to Dr. Burt's initials are redacted. Those dates are also earlier than November 3, 1999.

18. Turning to page 1 of Appendix D, it is seen that the title of the project is "The effects of Hill's antioxidant diet on the development of age-dependent cognitive dysfunction and neuropathology in canines." This shows that the purpose of the testing at LRRI was to feed a diet containing antioxidants to older pets to study cognitive dysfunction.

19. In question 2 on page 2 of Appendix D, the scientific goals of the LRRI project were stated in non-scientific terms. As seen there, the explanation of the project suggests that older dogs are to be fed diets high in antioxidants and the effect on the development of age-dependent dementia, loss of memory and learning skills are to be

studied. These indications are the claimed methods of inhibiting the loss of learning ability or increasing the learning ability of an aging companion pet in need of such treatment.

20. As explained further in question 3 of Appendix D, dogs in an "antioxidant diet group" will receive a diet rich in antioxidants, such as Vitamin E, Vitamin C, etc.

21. Cognitive testing of the subject animals is also indicated on page 4 of Appendix D. As noted at the top of page 4, the IACUC Office is required to prepare a list of experimental procedures performed on animals during the project. It is noted under Other, that "Cognitive testing" will be carried out.

22. As part of our testing at LRRI we were required to issue yearly progress reports to different agencies which provided partial support of which one was the United States Army. A yearly progress report for this entity was created before November 3, 1999 is attached as Appendix E. Appendix E is a printout of a Microsoft Word document print screen view of the draft report file for this funding entity. The properties window of the Microsoft Word document indicate that creation date and the last modified dates are both earlier than November 3, 1999. A copy of the metadata of the Microsoft Word file is attached as the last page of Appendix E, with appropriate redactions.

23. The introduction on page 1 of Appendix E, consistent with Appendix D, explains that the effect of antioxidants on age-dependent cognitive decline is to be studied in dogs. The introduction states that "a broad spectrum of antioxidants will be added for dietary enrichment."

24. Appendix E explains that the project at LRRI was a 3 year design. The document of Appendix E represents a progress report after the first year.

25. The entire progress report is included in Appendix E. However, attention is drawn to page 4, the second to the last paragraph, where it is reported that

antioxidants were considered for inclusion in the diets. Such antioxidants include Vitamin C, Vitamin E acetate, beta-carotene, carnitine, and lipoic acid.

26. On page 5 bridging to page 6 of Appendix E, we explain that the "final intervention diet" was formulated to be the basal diet plus addition of other components. The other components included carrots, spinach, tomato pomace, grape pomace and citrus pulp. As particularly relevant to the claims, the final intervention diet was also formulated to contain Vitamin E, Ascorbic acid (Vitamin C), Selenium, Carnitine, and Lipoic acid. Appendix E thus shows that we conceived the idea of feeding an intervention diet containing Vitamin E, Vitamin C, Carnitine and Lipoic acid to aged dogs in order to determine the effects of the antioxidants on age-dependent cognitive decline.

27. As discussed above, a diet containing the four antioxidants Vitamin E, Vitamin C, L-carnitine and Lipoic acid was formulated on a date earlier than November 3, 1999. See the discussion above of Appendices A, B and C. Manufacture of the diet to be used in the LRRI testing corroborates the conception of the idea given in Appendix E, that idea being to feed dogs a diet containing the four claimed antioxidants to inhibit the loss of learning ability or increase their learning ability.

28. I hereby declare under penalty of perjury that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: JUNE 2, 2008

Steven C. Zicker, DVM, PhD

EXHIBIT A

CONFIDENTIAL

Hill's® Pet Nutrition, Inc. - Science and Technology Center

Make Sheet for Grain Mix

For production at

Requested by Steven Zicker

Product Number:	Product Name:
Formula Number: 15658	Formula Description: NMSENIOR TEST+CARNIT/TAU
Variable Number:	Variable Description:
Project Number:	Project Description:
Job Code:	Job Code Description:

Total lbs finished product:	6,000.00
Process % of finished product:	94.65
Pounds from this process:	5,679.18
Pounds per batch:	1,893.06
Batches required:	3.00
Flow rate from this process:	0.00

Make Sheet Comments:

Package in 40 lb white bags
Cheryl to provide labels
Ship to New Mexico/Toronto as per Cheryl
Save 1x40lb bag for analysis

Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch	
2121.4	Yellow Corn, Whole	62.485	66.015	3749.100	1249.70	[redacted]
2322.4	P.Meal RA Perdue	11.105	11.732	666.300	222.10	[redacted]
2155.4	Rice, Brewers Milled	5.000	5.282	300.000	100.00	[redacted]
2175.4	Soybean Mill Run	4.500	4.754	270.000	90.00	[redacted]
2124.4	Corn Gluten Meal	2.600	2.747	156.000	52.00	[redacted]
2193.1	Egg, Compacted	1.000	1.056	60.000	20.00	[redacted]
9483.1	Spinach Fl LaBud	1.000	1.056	60.000	20.00	[redacted]
9485.1	Citrus pulp, Trp	1.000	1.056	60.000	20.00	[redacted]
9486.1	Tomato Pomace WE	1.000	1.056	60.000	20.00	[redacted]
9487.1	Grape Pomace,AGR	1.000	1.056	60.000	20.00	[redacted]
9488.1	Carrot Dr LaBudd	1.000	1.056	60.000	20.00	[redacted]
2103.4	Flaxseed, Ground	0.600	0.634	36.000	12.00	[redacted]
PRE	Grain Premix	2.363	2.496	141.780	47.26	
	TOTALS	**94.653**	**100.000**	**5679.18**	**1893.06**	

1 - 16.7 lbs Ref Steve Z.

LS+[redacted]

JH[redacted]

Approved: _____ Date_____

EXHIBIT B

printed by Steven Zicker, [redacted]

CONFIDENTIAL

D
AR

Hill's® Pet Nutrition, Inc. - Science and Technology Center

Make Sheet for Grain Premix

For production at

Requested by Steven Zicker

X135
Inline Enrobe

Product Number: 100219
Formula Number: 16668
Variable Number: 0307
Project Number: 100219
Job Code: 0307

Product Name: NMSR TEST
Formula Description: NMSENIOR TEST+CARNIT/TAU
Variable Description:
Project Description:
Job Code Description:

collect As much as possible

Total lbs finished product:	6,000.00
Process % of finished product:	2.36
Pounds from this process:	141.78
Pounds per batch:	47.26
Batches required:	3.00
Flow rate from this process:	0.00

Make Sheet Comments:

Package in 40 lb white bags
Cheryl to provide labels
Ship to New Mexico/Toronto as per Cheryl
Save 1x40lb bag for analysis

Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch
2282.4	Potassium Chloride	0.464	19.636	27.840	9.28
2208.4	Dicalcium Phosphate	0.368	15.573	22.080	7.36
2271.4	Choline Chloride, 60%	0.260	11.003	15.600	5.20
9624.4	Carnitine 10% NF	0.257	10.876	15.420	5.14
9287.1	VITAMIN E 50 ADS	0.241	10.199	14.460	4.82
2261.4	Myvaplex 600P Glyceryl Monostearate	0.200	8.464	12.000	4.00
2269.4	Salt, Iodized	0.179	7.575	10.740	3.58
2201.4	Calcium Carbonate	0.100	4.232	6.000	2.00
2352.4	Vit 2352-k9 dry	0.065	2.751	3.900	1.30
2206.4	Taurine	0.053	2.243	3.180	1.06
9061.1	L-Tryptophan	0.044	1.862	2.640	0.88
2305.4	2305 Mineral Mix	0.042	1.777	2.520	0.84
2237.1	Ascorbic Acid Coated (EC)	0.029	1.227	1.740	0.58
9429.1	Naturox Plus Dry	0.020	0.846	1.200	0.40
2207.1	Magnesium Oxide (MAGOX)	0.017	0.719	1.020	0.34
9517.1	a-lipoic acid	0.014	0.592	0.840	0.28
9516.1	Se-Yeast	0.010	0.423	0.600	0.20
	TOTALS	2.363	100.000	141.78	47.26

Stay-C 352 .029 1.227 1.740 [redacted] 0.58

Approved: _____ Date_____

LS+ OKEP
KM

EXHIBIT C

CONFIDENTIAL

Hill's® Pet Nutrition, Inc. - Science and Technology Center

Make Sheet for Topical

For production at

Requested by Steven Zicker

Product Number:
Formula Number: **16668**
Variable Number:
Project Number:
Job Code:

Product Name:
Formula Description: **NMSENIOR TEST+CARNIT/TAU**
Variable Description:
Project Description:
Job Code Description:

Total lbs finished product: **6,000.00**
Process % of finished product: **5.35**
Pounds from this process: **320.82**

Make Sheet Comments:

Package in 40 lb white bags
Cheryl to provide labels
Ship to New Mexico/Toronto as per Cheryl
Save 1x40lb bag for analysis

Ingredient Listing

Ingr Code	Ingredient	% of Diet	% of Mix	Total lbs	lbs Batch
2179.4	Soybean Oil, Crude Degummed	2.247	42.024	134.820	798.53 44.9 lb
8123.4	Grease, Choice White	2.100	39.274	126.000	745.49 42 lb
8271.4	Optimizer LDPE H-Plus	1.000	18.702	60.000	354.04 20 lb
	TOTALS	5.347	100.000	320.82	1893.06 106.9 lb

Approved: _____ Date_____

EXHIBIT D

APPROVED COPY

[redacted]

Refer to Instructions for Completing Form 8225
This Form Must Be Typed Please Number All Pages

For Office Use Only

Three-year Approval Period:

Date Received [redacted].
Protocol/Proposal# [redacted].
Approval Date [redacted].

Lovelace Respiratory Research Institute
Institutional Animal Care and Use Committee
PROTOCOL/PROPOSAL TO USE VERTEBRATE ANIMALS IN RESEARCH, TESTING, OR INSTRUCTION
(Form 8225)

__X__ New Protocol/Proposal
_____ Renewal Protocol/Proposal;
(Previous Protocol/Proposal #_____.)

Principal Investigator/Study Director
(Must be a Staff Member or Postdoctoral Fellow) Bruce A. Muggenburg_____.

Title: Senior Scientist_____.

Division/Unit Administering Accounts: Toxicology_____.

Mailing Address: _____North __X__South _____Other

Telephone # (505) 845-1119_____ FAX # (505) 845-1198_____.
E-mail address: bmuggenb@lrri.org_____.

Other Contact Person(s):
Name: F. F. Hahn Position: Study Pathologist
Name: Position:

Project/Title: (Enter Same Title as Grant Applications)

Title: The effects of Hill's antioxidant diet on the development of age-dependent cognitive dysfunction and neuropathology in canines.

Funding Agency: Hill's Pet Nutrition_____ Currently Funded? Yes___No__X_.

Present Grant/Contract Number (if known):_____

To which account should Animal Care charges be billed to?

SUBMIT COMPLETED FORM TO: INSTITUTIONAL ANIMAL CARE & USE COMMITTEE, ATTN: IACUC Office, LRRI SOUTH. TELEPHONE: 845-1025. ALLOW 2-4 WEEKS FOR APPROVAL.

DESCRIPTION OF PROJECT (QUESTIONS 1 - 5)

1. Which of the following describes the type of animal use proposed in this application? (Check all that apply.)

   X Basic Research
   _____Applied Research
   _____Field Research
   _____Instruction or Training
   _____Testing (toxicology, etc.)
   _____Service (breeding, core facility, surveillance)
   _____Other (Specify _____)

2. How would you explain to a non-scientist the long-term or overall scientific goals of the proposed work?

Pet owners and veterinarians have long recognized that some older dogs develop behavior changes suggesting senility. Research done at this Institute and elsewhere have shown that dogs do develop age-dependent dementia and that the neuropathology and loss of memory and learning skills are similar to these changes in aged humans. Because the dog appears to be a good model for human brain aging, we propose to evaluate two interventions that may delay the onset of age-dependent dementia. The two interventions are: 1) a diet high in antioxidants in the form of Vitamins E and C, and food products with naturally occurring anti-oxidants, and 2)a program of enriched learning and physical exercise experiences.

3. How would you explain to a non-scientist the specific objectives of the proposed work?

The dogs will be divided into four groups. The objective for each group is given below:

1. Twelve dogs will be in the control group and will be fed a normal balanced diet. Each dog will be housed individually in a run and will be evaluated for cognitive function and physical health once a year. This group is expected to have normal aging.
2. Twelve dogs will be in the antioxident diet group. These dogs will be treated like the dogs in group 1 but will receive the diet rich in antioxidents (Vitamins E and C, etc.). This group is expected to age with smaller losses in learning and memory abilities than the dogs in group 1.
3. The twelve dogs in this group will receive the normal balanced diet but will be housed with a pen mate and will be given mental and physical enrichment. We hypothesize this group will have smaller losses in learning and memory than group 1 dogs because exercise has been shown to have an antioxidant effect.
4. The twelve dogs in this group will receive the high antioxidant diet and the mental and physical enrichment. We expect this group to have significantly smaller aging changes than any of the other groups.

   (This study is one half of a larger study. Only 24 dogs are in this study and the other 24 dogs are in protocol FY98-001.)

5. How would you explain to a non-scientist the ways the proposed animal use might benefit human or animal health or the environment,

the advancement of knowledge, or the good of society?

This study will test the hypothesis that at least part of the aging process is due to oxidant damage to the brain, damage that leads to the death of neurons and the subsequent loss of memory and learning functions. If these interventions prove successful over the course of this study, it will provide a way in which both humans and their companion animals (at least dogs) can improve the quality of life during aging.

APPROVED COPY

The IACUC Office is required to prepare a list of experimental or instructional procedures performed on animals for the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

5. From the list below, check all experimental or instructional procedures that will be performed on the animals requested in this application. All procedures checked below must be explained in Question 8. (Check all that apply.)

_____Addiction or addiction withdrawal
_____Amputation
__X__Anesthesia
_____Antibody production
_____Ascites production
_____Behavior modification/operant conditioning
_____Biopsy
__X__Blood collection
_____Breeding
_____Burn
_____Cannulation
_____Catheterization
_____Capture of wildlife
_____Cardiac puncture
_____Dental procedure
__X__Environmental manipulation
__X__Euthanasia
__X__Food/water manipulation
_____Gavage
_____Immunization, experimental
_____Immunosuppression
_____Implant
_____Injury/trauma
_____Injection
_____Inhalation exposure
___nose-only___whole body
_____Inoculation, experimental
_____Irradiation
_____Irritation, experimental
_____Lavage
_____Myocardial infarction
_____Noxious stimulus
_____Obesity, experimental
_____Observation only (no manipulations)
_____Organ/system failure or dysfunction, experimentally induced
_____Paralysis, experimentally induced
_____Peritoneal lavage
_____Prey, animal
__X__Radiography
_____Restraint
_____Sensory dysfunction
_____Sepsis induction
_____Stress
_____Stroke
_____Surgical procedure, non-recovery
_____Surgical procedure, recovery
_____Toxicity test
_____Transplantation
_____Tumor growth, experimentally induced
__X__Other Cognitive testing

LOVELACE RESPIRATORY RESEARCH INSTITUTE
ANIMAL CARE AND USE COMMITTEE PROTOCOL DISPOSITION FORM

[redacted]

Protocol/Proposal #: [redacted] Investigator: B. A. Muggenburg Review Date: [redacted]

__ Proposal XX New Protocol __ Annual Review __Three Year Resubmittal __ Other

Dear Dr. B. A. Muggenbug:

Your protocol, entitled, "The Effects of Hill's Antioxidant Diet on the Development of Age-Dependent Cognitive Dysfunction and Neuropathology in Canines", was considered by the Institutional Animal Care and Use Committee, and the following decisions were made:

XX The proposal/protocol/amendment** was approved as presented.

_____The protocol/proposal was not approved by the committee for reasons stated in the attached memorandum..

_____The annual report was approved for another year.**

_____The annual report was accepted and completion of the study noted. The file has been inactivated.

_____The contingencies outlined by the committee in its initial review have been satisfied and the protocol has received final approval.**

_____The committee recognizes no major problems or unacceptable practices in the protocol./proposal, however, some changes are needed or additional information is requested. The protocol can be approved when answers to the committee's questions or modifications to the protocol listed on the attached memorandum are received and approved by the chairman or his designee.**

_____The committee has serious questions and/or concerns about the research protocol. These are described in the attached memorandum. The amendment to the protocol cannot be approved until the committee reviews the response to the questions and/or concerns.

If you have any questions you may contact Dr. David Burt (845-1016) or Dr. Steve Rohrer (845-1049) and discuss your protocol.

Thank you,

D Burt

Date: [redacted]

Stephen R. Rohrer, PhD, Chairman, IACUC
or **David G. Burt, DVM, Attending Veterinarian

File: S1(L:)\ Group\AnimalS\DispForm.Doc

EXHIBIT E

Table of Contents

Introduction

The purpose of the current research project is to determine the effects of both antioxidants and environmental enrichment on age-dependent cognitive decline in a 3 year longitudinal design using beagle dogs. Dogs will undergo baseline screening of cognitive function and general health evaluation including clinical pathology and physical examinations. Magnetic resonance scans will be used to obtain in vivo measures of brain and cerebrovascular function. Each dog will be placed into one of four groups, which are counterbalanced with respect to cognitive ability, sex and age: (1) control group (2) enriched environment (3) dietary enrichment and (4) combined dietary and environmental enrichment. A broad spectrum of antioxidants will be added for dietary enrichment. The environmental enrichment condition will consist of additional cognitive experience and enriched sensory environment. Cognitive function, physical health and brain MR's will be monitored yearly to establish ongoing effects of the treatment. At the end of the study, detailed histological analysis of brain tissue will be correlated with cognitive function and MR measures of brain atrophy and cerebrovascular function to establish the effectiveness of the treatments on delaying or preventing the development of age-dependent neuropathologies.

Progress Report

In year 1 we proposed to collect baseline measures and to begin the intervention studies. Each of the baseline measures will be discussed separately below.

Evaluation of Health Status

The physical and neurological examinations for individual dogs have been completed and the general health of the dogs indicates no illnesses, sensory or motor impairments that exclude participation in the study.

Blood biochemistry profiles indicate that most dogs fall within the range of values considered normal for 51 individual blood biochemistry measures. No significant differences between the blood biochemistry profiles across treatment groups are evident except for corrected calcium levels indicating that dogs assigned to the enriched environment and antioxidant combination group have lower levels than the other 3 groups ($F(3,21)=3.387$ $p<.041$). However, considering the number of comparisons being made across the 51 measures, it is more than likely that this is a spurious difference.

Spontaneous Behavior Tests

Baseline testing of open field behavior is complete and includes measures of distance traveled, urination frequency, sniffing frequency, rearing and jumping frequency, time spent inactive and vocalization frequency. An analysis of variance comparing the 4 groups on these baseline values indicate no significant differences across treatment groups as shown in Table 1.

TABLE 1. ANOVA OF OPEN FIELD BEHAVIORS

		df	F	Sig.
DISTANCE	Between Groups	3	.173	.913
	Within Groups	20		
	Total	23		
URINE	Between Groups	3	.882	.467

	Within Groups	20		
	Total	23		
SNIFF	Between Groups	3	1.690	.201
	Within Groups	20		
	Total	23		
INACTIVITY	Between Groups	3	1.521	.240
	Within Groups	20		
	Total	23		
GROOM	Between Groups	3	1.319	.296
	Within Groups	20		
	Total	23		
REAR	Between Groups	3	2.216	.118
	Within Groups	20		
	Total	23		
VOCAL	Between Groups	3	2.036	.141
	Within Groups	20		
	Total	23		
JUMP	Between Groups	3	1.143	.356
	Within Groups	20		
	Total	23		

Pretraining in testing apparatus

Reward Approach Learning. This task is intended to be a pretraining phase to teach dogs to work in the test apparatus. Dogs are taught to look for a food reward in one of 3 recessed food wells rather than to rely upon olfactory cues. Dogs are given 10 trials per day and are required to meet one of two criteria: 9/10 correct on one day or 8/10 correct on 2 consecutive days. The score assigned to individual animals is the sum of errors across all days of testing up to and including the day when criterion was met. Of 28 dogs originally screened, 24 dogs were able to learn the reward approach task and will be used for the long term study. No significant group differences were noted on this measure of skill-learning (Table 2)

Object Approach Learning. This task is used to train dogs to manipulate objects on the presentation tray in the test apparatus. As reward approach learning, no significant differences were found across the four treatment groups at baseline (Table 2).

Visual Discrimination Learning: This task measures habit formation in dogs and involves presenting dogs with two objects simultaneously. Only one of these objects is consistently associated with a food reward. For dogs to be able to learn this problem they must also have sufficient visual acuity to differentiate the two objects and ensures that sensory ability is not compromised in individual dogs. No significant differences across treatment groups on this task were found (Table 2)

Reversal Learning: This task procedes in an identical manner as visual discrimination learning, using the same 2 objects, however, the reward contingencies are reversed. Dogs are required to inhibit a response to the previously correct object and to learn to respond to the previously incorrect object. All dogs were able to learn the task and no significant group differences on this baseline measure were found (Table 2).

Training on the Spatial Memory Test

All dogs have completed testing on a spatial delayed non match to position task and found the problem particularly difficult to learn. Four of 24 dogs were able to meet criterion and no significant differences exist between the four treatment groups (Table 2).

Training on the Object Recognition Memory Test

All dogs have completed testing on an object recognition non match to sample task and 15 of 24 dogs were able to learn the problem. Of the 15 dogs that met criterion, some dogs were able to remember information for as long as 150 seconds. No significant differences exist between the four treatment groups (Table 2).

Baseline Magnetic Image Resonance Scans

Baseline MRI's have been conducted on all 24 dogs. All dogs tolerated the procedures well and suffered no adverse side effects. The figure below illustrates coronal sections from 2 dogs that are typical of each treatment groups. MR scans indicate no gross abnormalities in any of the dogs included in the study. The anatomical and cerebrovascular analyses are underway and are being conducted by Dr. Lydia Su at the University of California as per our protocol. Procedures are being standardized to be applied to individual dogs and across scanning sessions.

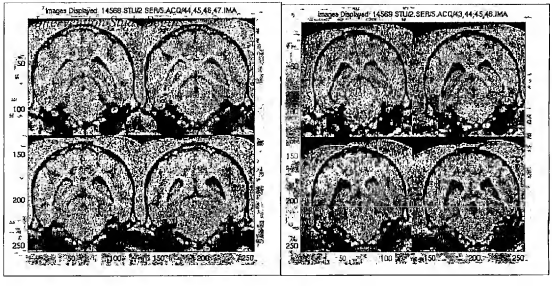

Figure 1. MR images are presented for two representative dogs illustrating no gross abnormalities. Note that the dog on the right appears to have larger ventricles than the dog on the left, which will be quantified using volumetric analyses.

Group Assignments: To balance the treatment groups, the error scores from individual dogs were summed across all of the baseline cognitive tasks (reward and object approach learning, visual discrimination and reversal, object and spatial memory). Males and females were split into 2 groups and dogs were given a ranking from 1 to 12 based upon an ascending series of the individual error scores. Dogs were assigned to each group counterbalanced for sex and for rankings on the cognitive test scores. The table below illustrates that placing dogs in treatment groups in this manner results in equally matched groups of dogs across all tasks, illustrated by the lack of significant F values for all tasks.

TABLE 2. ANOVA FOR TESTING FOR GROUP DIFFERENCES IN BASELINE COGNITIVE TEST SCORES

		Sum of Squares	df	Mean Square	F	Sig.
Reward Approach	Between Groups	207.792	3	69.264	.826	.495
	Within Groups	1677.167	20	83.858		
	Total	1884.958	23			
Object Approach	Between Groups	10.792	3	3.597	.548	.655
	Within Groups	131.167	20	6.558		
	Total	141.958	23			
Visual Discrimination	Between Groups	928.833	3	309.611	2.137	.127
	Within Groups	2897.000	20	144.850		
	Total	3825.833	23			
Reversal Learning	Between Groups	2981.667	3	993.889	1.617	.217
	Within Groups	12295.667	20	614.783		
	Total	15277.333	23			
Object Recognition Memory	Between Groups	4374.792	3	1458.264	.191	.901
	Within Groups	152307.833	20	7615.392		
	Total	156682.625	23			
Spatial Memory	Between Groups	21337.518	3	7112.506	2.209	.122
	Within Groups	57965.300	20	3220.294		
	Total	79302.818	23			

Exercise and Environmental Intevention: All dogs are now either housed singly or in pairs depending upon the experimental condition. Dogs have begun an exercise program that involves walking on a leash for period of 20 minutes, twice per week. The first series of additional learning experiences is underway with dogs being trained on a landmark discrimination task. Dogs are being tested 5 times per week.

Antioxidant Diet Intervention: A commercial, senior formula, dog food was selected for the basal diet. The basal formula meets all AAFCO (American Association of Feed Control Officials) recommendations for a food to be fed to domestic dogs. A variety of ingredients were considered for fortification of the diet.

Raw ingredients considered were screened for carotenoid, flavinoid, and ORAC (oxygen radical absorbance capacity) content. From these data the 5 ingredients with the highest combined ORAC/carotenoid/flavinoid content that were economically & legally feasible for inclusion into a commercial feed were selected. The components selected were: spinach, carrot granules, grape pomace, tomato pomace, and citrus pulp. Each of these was included at a 1% inclusion rate into the diet as a direct substitute for corn.

Purified or synthetic ingredients considered for inclusion were: vitamin C (Stay-C [Roche], vitamin E acetate, beta-carotene, carnitine, and lipoic acid. Selenium yeast was also included since selenium bioavailability in common ingredients for pet foods is low (20-30%) A range for the rate of dietary inclusion was extrapolated from other mammalian studies based on dose per body weight or dose per body weight to the ¾ power. In the case where insufficient data in other species existed to convert by body weight a best estimate was made.

Experimental foods
Variations of the basal diet were made based on the above ingredients. All numbers are in ppm per dry matter of food.

Table 3. Targeted vitamin level additions in antioxidant diets

Treatments	Vit E	Vit C	β-carotene	Selenium	Flavonoids	Carotenoids	Lipoate
1.Ctrl	50	--	--	.6		X	
2.half vit.	250	100	6	.66		X	
3.full vit w/o lipoate	500	200	12	.66		X	
4.full vit + frt/veggie	500	200	12	.66	X	X	
5.fruit/veggie only	50	30	0.2	.6	X	X	
6.full vit w/ lipoate	500	200	12	.66		X	100

These 6 diets were then put into a feeding trial that utilized 48 dogs (8 dog per group) at Hills Pet Nutrition in house. All dogs were fed basal diet for 4 weeks prior to intervention with food. A control group was maintained on basal diet for the duration of the study. Dogs were blocked into groups based on age and sex. Dogs were fed intervention diets for 4 weeks.

Table 4. Mean post-antioxidant measurements as affected by diet.

Diet	**ORAC-total**	**Sera Vit E**	**Sera Vit C**	**Urinary isoprostane -Cr**	**Urinary 8-OH-dG-Cr**
	μM	μg/ml	μg/ml	ratio	ratio
1. Senior control	3683	58.9	20.7	20.8	.34
2. Senior + half vitamin	4327	70.8	20.1	16.8	.64
3. Senior + full vitamin	4726	92.1	17.5	21.3	.45
4. Senior + full vitamin + fruit/veg	4447	88.2	46.8	19.7	.42
5. Senior + fruit/veggie only	4037	61.9	32.6	27.5	.44
6. Senior + full vitamin + lipoate	5066	94.2	28.1	15.3	.25
Typical ranges for humans:	**3300-5000**	**6.5 - 17.2**	**7 - 26.5**	**0-34**	**0-0.74**

Based upon our findings, these data would suggest that the half and full inclusion rates of vitamins C, E , and Selenium were efficacious in improving antioxidant status as measured by changes in ORAC and sera E. Although it was our intention to evaluate the efficacy of beta-carotene in this study, because of high beta-carotene losses in the first formulations and poor dispersion in the topicals with the second round of formulations, we were unable to evaluate beta-carotene. The addition of fruits and vegetables had a positive effect on serum vitamin C concentration. The addition of lipoate to the supplemental vitamin mix trended towards significance in reducing urinary isoprostane and 8-OH-2'deoxyguanosine. Utilizing the above information it was concluded that a mix of antioxidant ingredients and pure vitamin additions would provide the best matrix for increasing protection from free radical damage without any adverse effects in the target species. Therefore the final intervention diet was formulated to be comprised of the basal diet plus additions of the following ingredients:

1% each of carrots, spinach, tomato pomace, grape pomace, citrus pulp

Vitamin E

Ascorbic acid as Stay-C

Selenium
Carnitine
Lipoic acid

Efficacy of Diet Intervention

We have collected blood samples from all the control diet dogs and 5 of the intervention diet dogs to assay vitamin E levels after 3 months of being fed the diet rich in antioxidants while on study. Dogs receiving the antioxidant diet show a significant increase in blood levels of vitamin E ($t(5)=2.824$ $p<.048$).

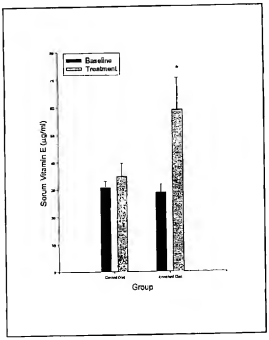

Figure 2. The group mean of dogs receiving the control diet are compared to the group mean of dogs receiving the antioxidant diet. The antioxidant diet results in a significant increase in serum levels of vitamin E (* indicates $p<.05$).

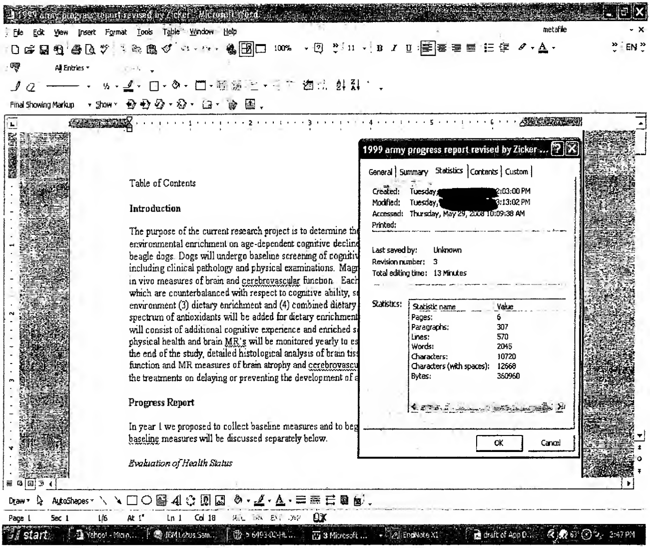

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Table of Contents
Introduction
The purpose of the current research project is to determine th
environmental enrichment on age-dependent cognitive declin
beagle dogs. Dogs will undergo baseline screening of cognitiv
including clinical pathology and physical examinations. Mag
in vivo measures of brain and cerebrovascular function. Each
which are counterbalanced with respect to cognitive ability, s
environment (3) dietary enrichment and (4) combined dietary
spectrum of antioxidants will be added for dietary enrichment
will consist of additional cognitive experience and enriched s
physical health and brain MR's will be monitored yearly to es
the end of the study, detailed histological analysis of brain tis
function and MR measures of brain atrophy and cerebrovascu
the treatments on delaying or preventing the development of a
Progress Report
In year 1 we proposed to collect baseline measures and to beg
baseline measures will be discussed separately below.
Evaluation of Health Status
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